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Research Article

Enumeration of Cellulase Producing Bacteria from Forest Samples and Optimization of Cellulase Production for Saccharification

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ABSTRACT

In the present study the bacterium was isolated from decaying wood samples of Western Ghats forest and identified as Bacillus cereus by 16S r RNA sequence which was used for cellulase production. The basal media (Czapek Dox) was optimized with respect to pH, temperature, carbon source (Typha latifolia- aquatic plant) and nitrogen source (yeast extract) by using Response Surface Methodology (RSM). Maximum exoglucanase (Fpase) production was recorded at pH 7.0, 45^{0}C temperature, 9% carbon source and 0.7% nitrogen source. Crude cellulase enzyme from Bacillus cereus had effectively saccharified biomass into reducing sugar, which shows that cellulase production from this strain can be effectively hydrolyse the lignocellulosic material for bioethanol production.

Key words: *Bacillus cereus; Exoglucanase; Typha latifolia; Response Surface Methodology;*

INTRODUCTION

Lignocellulosic biomass is available in plenty and is an economical renewable resource for bioconversion of biofuels. However, lignocellulosic materials are complexly packed to resist enzyme degradation[1]. A group of cellulase enzyme like endoglucanase, exoglucanase and β-glucosidase have different enzymatic action on hydrolysis of biomass[2]. Several cellulase enzymes produced from microbes and bacteria have efficiency in bioconversion of biomass to valuable products[3]. Some of the cellulase producing microbes play crucial role in recycling of cellulose and biomolecules on earth. Because of tremendous industrial application of cellulase enzymes is growing at great rate ahead[4].

Various cellulosic materials are used for enzyme production by fermentation process. Most routinely agriculture substrates are used in biofuel industry which has raised a food versus fuel debate[5]. In current trend, biofuel production industries are focussed on marine algae and lignocellulosic materials, rather than first generation biomass[6].

Aquatic plants are a promising source of renewable energy, as they outweigh the economically constrained utilization of grains and vegetables on arable farmland[7].

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Since, aquatic plants produce huge tonnes of lignocellulosic biomass and indirectly effects on human. Using this biomass for biofuel production can be usually having advantage on nutrient balance in aquatic ecosystem[8].

The cost of biomass is the limiting factor in developing an economic process for cellulase production. This could be improved by using cheap lignocellulosic biomass for enzyme production[9]. *Typha latifolia* grows in wetland condition and have potential biomass, superior in chemical composition[10].

Therefore, this type of cellulosic waste can be utilized for[11] for cellulase production. Western ghats forest extended huge area about of 1,600 km parallel in 140,000 km² in a stretch towards of the peninsular Indian and one of the world's tenth hottest biodiversity hotspots, here a unknown number of microbes which could be important for exploring for potential applications for socio-economic development.

In the present work, cellulase producing bacterium, isolated from decaying wood, has been utilized for the optimization of cellulase production and was analysed for the maximal efficiency of saccharification of crude cellulase enzyme on biomass.

MATERIAL AND METHODS

2.1. Isolation

Soil and decaying wood samples used in this study were collected from Western Ghats region of Karnataka. 1g of sample was serially diluted in sterile distilled water then sample from 10^4 dilutions were spread on Carboxy-Methyl cellulose (CMC) agar medium plate pH 6.8. The media consisted of (in g/L) 10 CMC, 1 K_2HPO_4, 0.5 $MgSO_4$, 0.5 NaCl, 10 peptone and 0.25 $(NH4)_2SO_4$ incubated at 37 ^{0}C for 48 hours. The highest zone of clearance around bacterial colony was selected for identification[1] and optimization of cellulase production by submerged fermentation and its application on biomass saccharification.

2.2. Molecular identification and phylogenetic analysis

A bacterium culture was overnight grown on nutrient agar medium and DNA extracted using spin microbial DNA kit (MACHEREY-NAGEL GmbH & Co, Germany). The gene encoding bacterial 16S rRNA was amplified through PCR with primers, 27F-5′ AGAGTTTGATCMTGGCTCAG 3′ and 1492R-5′ TACGGYTACCTTGTTACGACTT 3′. Using codon code software consensus sequence was generated[12]. Near similar database was performed using blast analysis and phylogenetic tree was build by MEGA software.

2.3. One variable at a time (OVAT)

Prior to optimization of exoglucanase enzyme production was screened using the following basal media -

1) Mandel and Reese medium (g/L): peptone (1g); urea (0.3g); KH_2PO_4 (2g); $CaCl_2$ (0.3g); $(NH4)_2SO_4$ (1.0g); $MgSO_4.7H_2O$ (0.3g); $MnSO_4.H_2O$ (0.016g); $FeSO_4.7H_2O$ (0.005g); $CoCl_2$ (0.002g) and $ZnSO_4.7H_2O$ (0.014g).

2) Minimal medium (g/L): KH_2PO_4 (0.04g), K_2HPO_4 (0.1g), Na_2HPO_4 (0.10g), NH_4SO_4 (0.008g), $MgSO_4$ (0.02g), (NH_4) SO4 (0.04g), $CaCl_2$ (0.027g).

3) Bushnell Haas medium (g/L): $MgSO_4$ (0.2g), Calcium chloride (0.02g), KH2PO4 (1g), K_2HPO_4 (1g), (NH_4) (NO_3) (10g), $Fecl_3$ (0.05g).

4) Czapek Dox medium (g/L): except (sucrose), $MgSO_4$ (1g). KCl (0.5g), NaNO3 (2g $FeSO_4$ (0.01g), K_2HPO_4 (1g).

Five different lignocellulosic materials were used as carbon sources for production of exoglucanase enzyme, *Typha latifolia* (TL), *Baccopa monnieri* (BM), *Alternanthera sessilis* (AS), *Eichhornia crassipes* (EC) and *Naja gudalapensis* (NG). All the substrates were ground to fine particle size less than 5-10 mm. processed samples were thoroughly rinsed with distilled water and allowed to dry at 60 ^{0}C, further dried substrates was used along with Czapek Dox media (since it reported highest cellulase production), inoculated with 4% (v/v) inoculum size and incubated at 37^{0}C on incubator shaker at 120 rpm. After fermentation, broth was centrifuged and supernatant used as cellulase production. The lignocellulosic substrate produced at

higher enzyme activity was considered as suitable substrate for optimization studies.

In order to optimize cellulase production, Czapek Dox medium was prepared with 1% *Typha latifolia* (w/v) for production of cellulase. Further, to enhance enzyme production, different nitrogen sources like beef extract, yeast extract, peptone and sodium nitrate at 0.1% (w/v) were used with the substrate Similarly, effect of pH (2 to 10), temperature (25 to 65 ^{0}C) and inoculum size (2 to 10%) was optimized for cellulase production (supplementary information). All experiments were performed in triplicate.

2.4. Determination of FPase activity

Fpase activity was measured as described by (Mandels and Weber, 1969). 0.5ml of crude enzyme with a Whatman filter paper strip of dimension 1.0×6cm (50mg) was placed into test tube. 0.5 ml of Sodium-Citrate buffer was added (0.05M pH 4.8) and then reaction mixture was incubated for 30 minutes at 37 ^{0}C. About 3ml of 3.5 -Dinitrosalicylic acid reagent was added and boiled for 5 minutes. Absorbance was read at 540 nm.

2.5. Response surface methodology

In order, to optimize the selected processing conditions CCD (2^k) model is shown in Table 1.

Second order polynomial regression equation (Eq1) was used to assess interaction among the selected independent variables such as: of pH (x_1); temperature (x_2); substrate (x_3) & nitrogen (x_4). The model predicted Fpase activity compared against experimental observed value. Eq1 denotes all interaction terms used to calculate predicted response.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{55} x_5^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 \quad \boldsymbol{Eq}\ (1)$$

Where, Y: predicted Fpase (U/ml) activity; β_0 is regression coefficient of the model; $\beta_1, \beta_2, \beta_3$: linear effects of independent variables, $\beta_{11}, \beta_{22}, \beta_{33}$: square effects of independent variables and $\beta_{12}, \beta_{13}, \beta_{14}, \beta_{23}, \beta_{24}, \beta_{34}$ indicates interaction effects of selected independent variables. The CCD model data was analysed with Statistic 13.4 (TIBCO Software Inc. Palo Alto, CA, USA). The statistical inference was drawn based on analysis of variance (ANOVA) & *F*-test, the quality of second order polynomial regression equation was assessed by regression of coefficient (R^2) value. Further, the model predicted optimal levels of independent variables were tested in triplicates, the observed value compared with predicted value.

2.6. Enzyme production by submerged fermentation

Cellulase production by *Bacillus cereus* was performed in submerged fermentation in Czapek Dox broth medium with 9% *Typha latifolia* as the sole carbon source. 50 ml of Czapek Dox broth medium was taken into 125 ml Erlenmeyer flask and autoclaved at 121^{0}C for 15 minutes at 15 psi pressure. After sterilization, the flasks were kept in room temperature for cooling and 2 ml of spore suspension (McFarland standard No. 1) was inoculated into fermentation flasks, incubated at 45 ^{0}C with agitation speed of 120 rpm for 24 hours of fermentation period. After completion of the fermentation time, the broth was centrifuged at 8000 rpm for 10 minutes and the clear free extract obtained after centrifugation was used as crude cellulase enzyme.

2.6. Saccharification of biomass

Saccharification of biomass done according to Irfan et al.[14]. 20 ml of sodium citrate buffer (50 mM) at pH 4.0 was added by using 4.5% solid loading (pre treated *Typha latifolia* with 0.2% oxalic acid) with crude cellulase enzyme having 30 FPU/g. In the control setup, commercial cellulase (ONOZUKAR-10) enzyme activity of 1U/mg was incubated in shaking incubator speed of 120 rpm for 24 hours at 45 ^{0}C. After incubation, fermentation broth was centrifuged at 8000 rpm for 10 minutes. The supernatant was used for analysing reducing sugar. Saccharification (%) was calculated using formula

$$Saccharification\ (\%) = \frac{\text{Reducing sugar (mg/ml)}}{\text{Substrate used (mg/ml)}} \text{ X } 100$$

RESULTS AND DISCUSSION

3.1. Screening and microbial identification

Bacillus cereus isolated from decaying wood sample, identified by 16RNA gene sequence, showed an analogy of 99% to the Bacillus species. This was submitted to the gene bank and the Accession No accredited was MH590292 (Fig 1).

3.2. One variable at time (OVAT)

Carboxymethyl Cellulose (CMC 1%) in Czapek dox broth reported maximum exoglucanase activity. Hence, Czapek Dox media was used as a basal medium for optimization of exoglucanase production with several influenced factors like pH, temperature, carbon, nitrogen and inoculum concentration.

The present work is selective to seven carbon and four nitrogen sources for exoglucanase production. Among the carbon substrates in *Typha latifolia* growth extract, maximum enzyme production was noted at 0.72 IU. Waghmare *et al.*[15], reported the same in *Klebsiella* spp. with maximum FPU of 0.36 IU and 0.12 IU, from grass powder and sorghum husk, respectively. According to Kumar[1], enzyme production depends on amount of nitrogen supplementation. In the present study, peptone, beef extract, sodium nitrate and yeast extract acted as nitrogen sources which has been supported by Sreena and Sebastain[16]. Yeast extract and peptone showed a significant increase in the enzyme production by *Bacillus subtilis* as reported by Deka *et al.*[17], Here, we report yeast extract with highest exoglucanase production of (0.84 IU) (Supplementary information) similar with 3.76 IU by *Aeromanas bestiarum*[18].

Optimization was carried within a pH range of 2.0 to 10.0 for maximal enzyme production. A pH of 6.0 showed enhanced production of exoglucanase (0.66 IU). Achary and Chaudhry[19], reported at pH 6.0, *Bacillus licheniforms* produced 0.22IU and 0.25IU of exoglucanase enzyme from wheat straw and rice straw respectively.

Temperature is an important abiotic parameter which influences enzyme production. Our results indicate that the highest enzyme activity was obtained at 45 ^{0}C (0.87IU) (Supplementary information). Kato *et al.*[20], reported that optimum temperature for growth and cellulose degradation of *Clostridium straminisolvens* sp as 50-55^{0}C.

Cumulatively, Different inoculum concentration varied from 2 to 10% on the enzyme production. In our study, 6% inoculum size gave the highest enzyme activity of 0.71 IU, which is similar to Singh and Kaur[21], who reported an increase in the *Bacillus* inoculum size at 5% (v/v). Shankar and Isaiarasu[22], reported maximum cellulase production by *Bacillus pumilus* at 2% (v/v) inoculum size.

3.3. Optimization of significant medium components using central composite design (CCD).

RSM was instrumental in the optimization of Czapek Dox medium components. A total of 27 experimental runs in central composite design with four independent factors pH (x_1), temperature (x_2), substrate (x_3) & nitrogen (x_4) were tested to study their effect on exoglucanase production. A full factorial CCD was done with 4 factors and the results are shown in Table 1.

Run number 3 & 17 exhibited maximum exoglucanase production. The mean optimum levels for the independent variables were computed by statistical analysis of curvilinear contour plots and regression analysis. Finally, the predicted solutions by CCD model at optimum conditions were validated against the observed or experimental response.

On the basis of initial exoglucanase activity (U/ml), independent variables were selected and assigned at varying temperature range of 25 to 65 ^{0}C, pH range 4 to 10, substrate concentration 5 to 13% and yeast extract 0.4 to 1%. The selected factors were tested for optimal exoglucanase activity by a principle of design of experiments, where high

resolution CCD design enables to study interaction and analysis of selected four independent factors at five level full factorial designs as shown in Table.1. The responses of dependent variable were fitted with a second order polynomial regression shown as follows

$$Y = 5.53 - 0.29x_1 + 0.018x_1^2 - 0.018x_2 - 0.0003x_2^2 - 0.241x_3 + 0.0009x_3^2 - 2.65x_4 + 1.37x_4^2 - 0.002x_1x_2 - 0.004x_1x_3 - 0.029x_1x_4 + 0.0009x_2x_3 + 0.003x_2x_4 + 0.050x_3x_4$$

Eq (2).

The *p* value and F-test used to draw statistical significance of the CCD model tested. The selected independent variable (linear & quadratic terms) pH, temperature, substrate (TL), Yeast extract was found be statistically significant $p < 0.0001$, Table 2 shows significant model.

The coefficient of determination (R^2) for the CCD model was 0.96 indicating suitability of selected factors for the exoglucanase activity (U/ml). The 2D contour response plots were obtained indicating mean effects of selected independent variables on exoglucanase as shown in Fig.2 (a-f). The exoglucanase activity is profoundly influenced by pH, temperature, carbon and nitrogen. Further, the mean desirability profile of selected independent factors and inbuilt CCD model builder interface of Statistica 13.4 has give the predicted for optimal conditions as shown in Fig.3 (a-b). As per the desirability and CCD model builder prediction as shown in Fig 3. 0.204 (U/ml) exoglucanase activities were predicted at pH of 7, temperature of 45 ^{0}C, substrate at 9% and yeast extract at 0.7%. The predicted solution was validated by triplicate trials at specified condition and observed vs. predicted solution was compared. The observed exoglucanase production was found to be with standard deviation of 1.09±0.04 IU as that of predicted response.

The effect of pH on *Bacillus cereus* for exoglucanase production was examined at various ranges from pH 1 to 14. The enzyme production was found to increase up to pH 4.0 and decreased as pH increased with alkalinity. Nema *et al*.[23], reported that enzyme activity in *Bacillus cereus* increases with increasing pH till it reaches pH 4-5; then it decreases at pH 7-11. Later, Mawodze *et al*.[24], reported that cellulase enzyme of *Bacillus subtilis* were active in the pH range of 5-6.5. Otajevwo *et al*.[25], observed that optimum pH of 5.5 was the most suitable for cellulase production from *Bacillus circulans* strains. Similar study by Immanuel *et al*.[26], reported that enzyme activity of *Bacillus* spp was least at pH 5.0, but increased at pH 6 to 7 which support our results.

Maximum enzyme activity was obtained at temperature range of 25 to 85 ^{0}C (Table1), Optimum exoglucanase production increased with the temperature up to 65 ^{0}C and decreased linearly. Ray *et al*.[27], reported that *Bacillus subtilis* and *Bacillus circulans* show effective cellulase production at 40 ^{0}C. *Bacillus* K-12 isolated from filter paper wastes produced a cellulase component containing Avicelase, Xylanase, CMCase and Fpase at 50 ^{0}C[28]. Similar work was carried by Ladeira *et al*.[29], i.e. *Bacillus* sp cultivated at 50 ^{0}C in liquid culture containing sugarcane bagasse and corn steep liquor showed a maximum activity of Avicellase (0.83U/ml) and CMCase (0.29U/ml) within 120 and 168 hours of culturing time respectively.

In the present research, yeast extract scaled up the enzyme production which was supported by Abou-Taleb *et al*.[30], who concluded ho concluded cellulase production has enhanced by supplementing yeast extract. Paudal and Qin[31], however, utilized peptone as nitrogen source for *Bacillus subtilis*, which proved to facilitate cellulase production.

We aim to develop a low cost enzyme production system from various aquatic plants used as carbon source (supplementary information). Among those used, *Typha latifolia* (substrate) showed highest enzyme production at 9% (w/v). Shah *et al*.[32], reported that 7% substrate concentration of banana waste yielded highest cellulase activity.

The crude enzyme produced from *Bacillus cereus* was checked for saccharification of *Typha latifolia*, pre-treated with oxalic acid. After enzymatic hydrolysis, total sugar

obtained from crude cellulase enzyme and commercial cellulase enzyme (ONOZUKAR-10) was found to be 8.43 mg/ml and 12.35 mg/ml respectively. The saccharification rate calculated for crude cellulase enzyme and commercial cellulase enzyme (ONOZUKAR-10) was 18.7% and 27.44% respectively. Tandon *et al.*[33], reported 12.81% hydrolysis rate of NaOH + H_2O_2, pre-treated with pine needles. Singh *et al.*[34], focussed on alkali pre-treated bagass that released maximum reducing sugar of 5.7 mg/ml at a saccharification rate of 57%.

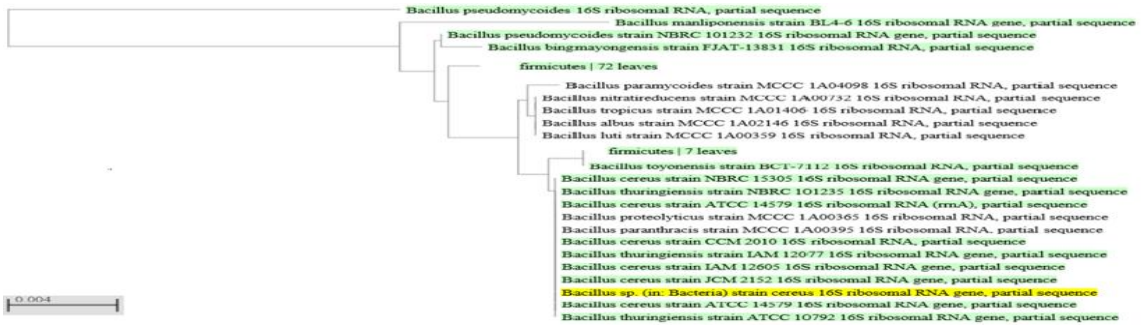


Fig 1. Phylogenetic tree of *Bacillus cereus* (MH 590292).

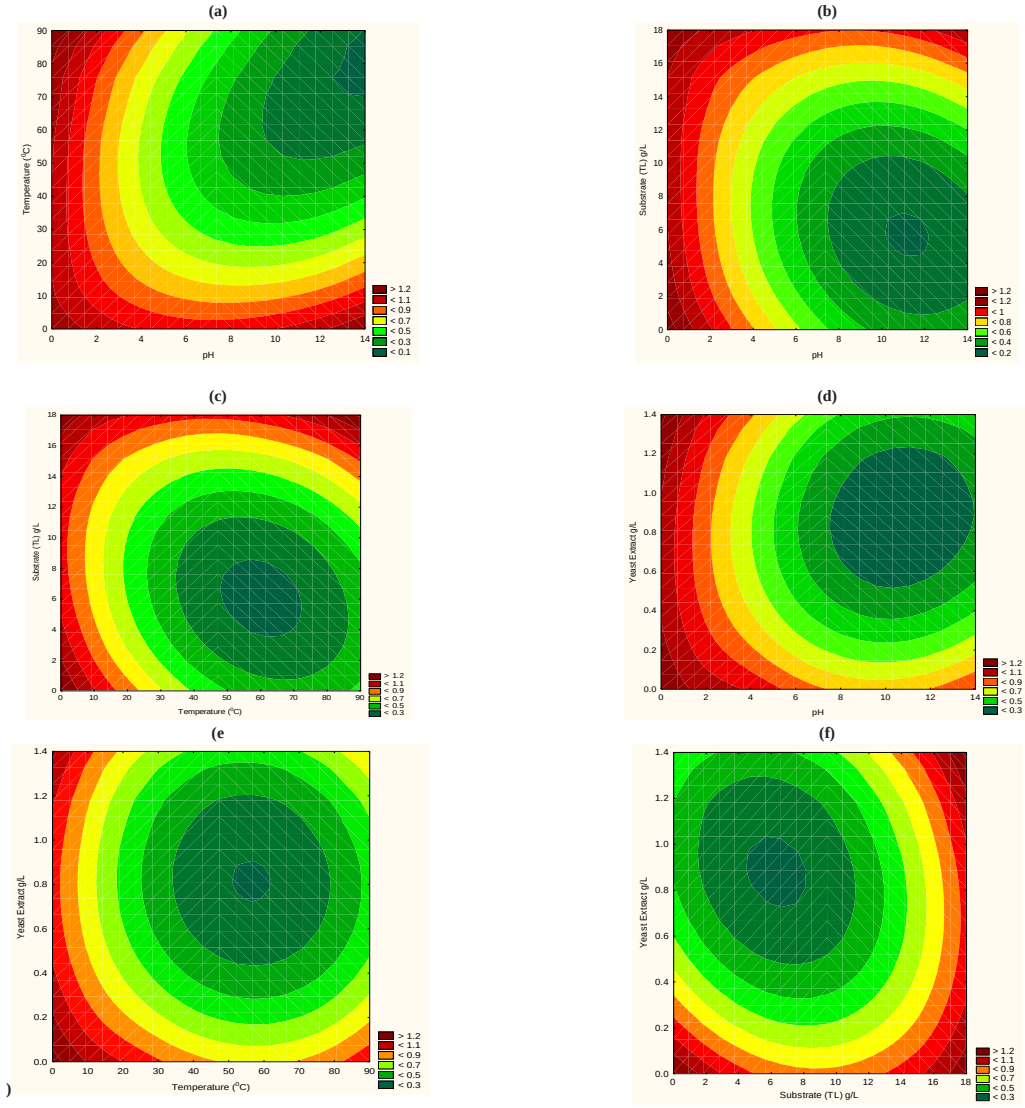


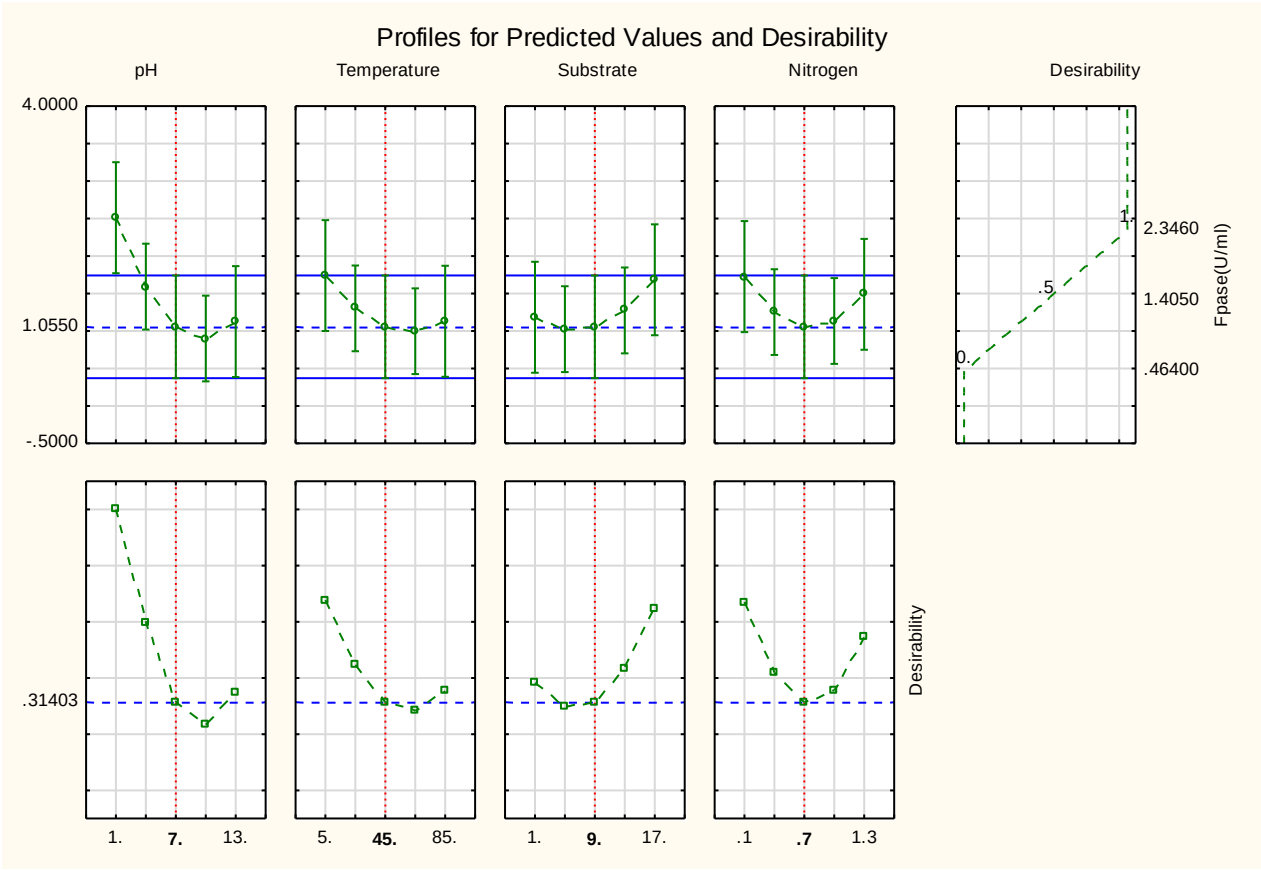
Fig 2. Two-dimensional contour response surface plots showing the mean effects independent variables and their interaction on exoglucanase activity (IU/ml). (a) Temperature vs pH (b) TL vs pH (c) TL vs Temperature; (d) YE vs pH (e) YE vs Temperature (f) YE vs TL

Table 1. Independent variables tested for optimization of exoglucanase (U/ml) production by central composite design (CCD)

Run No	pH (x_1)	Temp (x_2)	Substrate (TL) (x_3)	Yeast Extract (x_4)	Observed Exoglucanase activity (U/ml)	Predicted Exoglucanase activity (U/ml)
1	4	25	5	0.4	2.12	2.18
2	4	25	5	1	2.02	1.86
3	**4**	**25**	**13**	**0.4**	**2.29**	**2.11**
4	4	25	13	1	2.04	2.04
5	4	65	5	0.4	1.92	1.91
6	4	65	5	1	1.75	1.67
7	4	65	13	0.4	2.09	2.16
8	4	65	13	1	2.28	2.16
9	10	25	5	0.4	1.69	1.68
10	10	25	5	1	1.34	1.25
11	10	25	13	0.4	1.76	1.83
12	10	25	13	1	1.79	1.65
13	10	65	5	0.4	0.86	0.85
14	10	65	5	1	0.47	0.50
15	10	65	13	0.4	1.32	1.33
16	10	65	13	1	1.29	1.22
17	**1**	**45**	**9**	**0.7**	**2.29**	**2.43**
18	13	45	9	0.7	0.97	0.99
19	7	5	9	0.7	1.88	2.03
20	7	85	9	0.7	1.30	1.32
21	7	45	1	0.7	1.28	1.34
22	7	45	17	0.7	1.89	1.99
23	7	45	9	0.1	1.84	1.76
24	7	45	9	1.3	1.10	1.34
25C	7	45	9	0.7	1.07	1.06
26C	7	45	9	0.7	1.04	1.06

Table 2. Analysis of variance of exoglucanase activity (IU/ml)

Factor	Sum square	Degree of freedom	Mean square	F value	Probability
(1) pH (L)	3.115142	1	3.115142	138.8376	0.000001
pH (Q)	0.465495	1	0.465495	20.7465	0.000824
(2) Temperature(L)	0.744334	1	0.744334	33.1739	0.000127
Temperature(Q)	0.416949	1	0.416949	18.5828	0.001234
(3) Substrate TL (L)	0.637792	1	0.637792	28.4255	0.000240
Substrate(Q)	0.410232	1	0.410232	18.2835	0.001308
(4) Yeast extract (L)	0.268623	1	0.268623	11.9721	0.005332
Yeast extract(Q)	0.267859	1	0.267859	11.9381	0.005379
1L by 2L	0.306201	1	0.306201	13.6470	0.003537
1L by 3L	0.051482	1	0.051482	2.2945	0.158030
1L by 4L	0.011651	1	0.011651	0.5192	0.486195
2L by 3L	0.100423	1	0.100423	4.4757	0.058012
2L by 4L	0.005193	1	0.005193	0.2314	0.639881
3L by 4L	0.058715	1	0.058715	2.6169	0.134025
Error	0.246810	11	0.022437		
Total SS	6.228374	25			



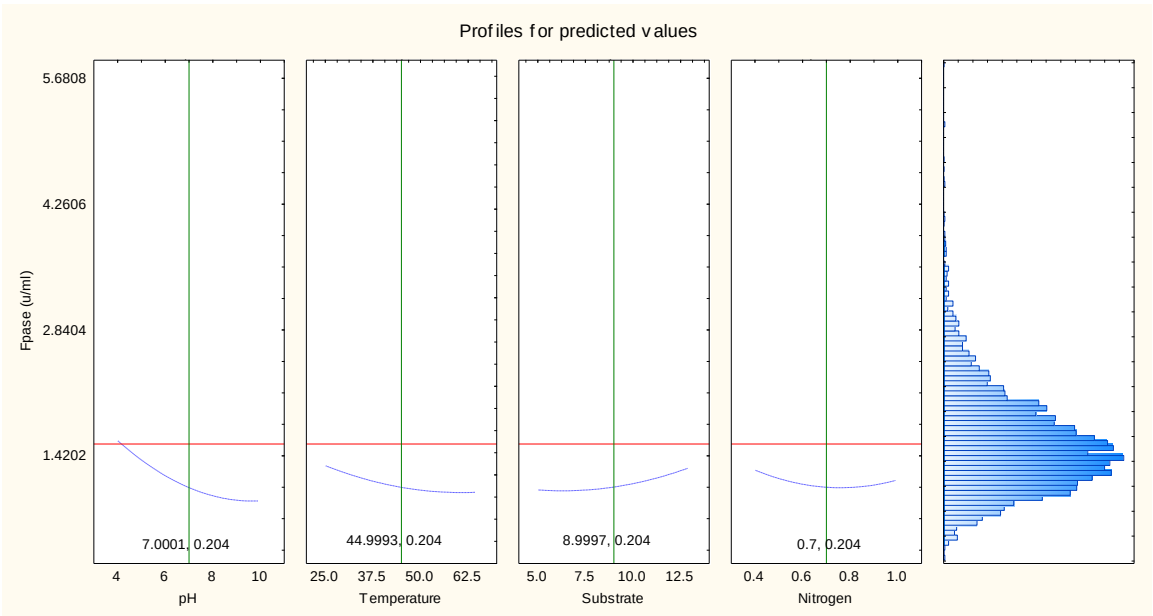


Fig 3. Desirability profile (a) & model builder prediction (b) for exoglucanase (IU/ml) production by *Bacillus cereus* (MH 590292) using response surface methodology

Supplementary information

Media	Exoglucanase activity U/ml
Czapek dox medium	0.34±0.02
Mandels and Reese medium	0.21±0.01
Minimal medium	0.18±0.01
Has medium	0.16±0.01
Carbon source	
Typha latifolia(TL)	0.72 ± 0.01
Bacopa monnieri(BM)	0.64 ± 0.03
Alternanthera sessilis(AS)	0.61± 0.02
Eichhornia crassipes(EC)	0.7 ±0.01
Naja guadalupensis(NJ)	0.54 ± 0.03
Carboxymethyl cellulose(CMC)	0.28±0.19
Filter paper	0.63±0.02
Nitrogen	
Peptone	0.63±0.01
Beef extract	0.58±0.01
yeast extract	0.84±0.02
Sodium nitrate	0.46±0.01
pH	
2	0.036±0.01
4	0.27±0.02
6	0.66±0.04
8	0.40±0.03
10	0.10±0.01
Temperature (^{o}C)	
25	0.67±0.01
35	0.79±0.02
45	0.87±0.01
55	0.61±0.02
65	0.49±0.02
Inoculum concentration(v/v)	
2	0.32±0.03
4	0.7±0.03
6	0.71±0.02
8	0.52±0.03
10	0.41±0.008

CONCLUSION

Western Ghats are rich sources of micro fauna having potential application for various industrial processes. The present source of microorganism from this enriched region has proved to be worthy of harvesting cellulase enzyme. Our research aim majorly to the industrial production of bioethanol. Therefore, we aim to provide cost effective solution in this field as it is a major global concern. Much higher and significant rates of saccharification have been evident in our study and it is anticipated that this might facilitate the production of biofuels.

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